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Role of Transforming Growth Factor β1 as Modulator of Endothelial L-Arginine/Nitric Oxide Signalling Pathway.

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Running Title: ***Regulation of L-arginine/NO pathway by TGF-β1.***

Transforming Growth Factor β (TGF-β).

TGF-β is one of the members of the TGF-β superfamily of growth factors, including activins/inhibins and bone morphogenic proteins (BMP). At least three TGF-β isoforms have been reported in mammals: TGF-β1, TGF-β2 and TGF-β3. Virtually all human cells produce TGF-β and express its plasma membrane receptors. Each isoform is coded by a different gene, in a tissue-specific and developmental-regulated manner. TGF-β1 mRNA is expressed in endothelial, haematopoietic and connective-tissue cells, TGF-β2 mRNA is expressed in epithelial and neuronal cells, and TGF-β3 mRNA in mesenchymal cells (1,3,25). The sequence homology is 70-80% between TGF-β isoforms and 30-40% with activins/inhibins and BMP. TGF-β1, 2 and 3 are highly conserved in mammals suggesting a critical biological function for each of these isoforms, acting as strong mediators of tissue repair through chemotaxis and angiogenesis stimulation and extracellular matrix generation (1,3,25).

TGF-β1 is a 112 amino acid polypeptide which forms a homodimer with both subunits linked with a disulfide bond. TGF-β1 is synthesized as a precursor of 391 amino acids whose amino acid sequence includes TGF-β1 and the propeptide latency-associated peptide (LAP) in the amino terminal. TGF-β1 is cleaved from LAP before the precursor is secreted from the cells, but it continues non-covalently bond to this propeptide. After being secreted, TGF-β1 is stored at the extracellular matrix as a complex formed between TGF-β1, LAP and the latent TGF-β binding protein (LTBP). The relation between TGF-β1 and LTBP through disulfide bond prevents the binding of TGF-β1 to its receptors. *In vivo*, TGF-β1 is released from the complex by the matrix glycoprotein thrombospondin 1 (TSP-1), which changes the conformation of LTBP (1,25).

TGF-β1 Signalling

Cellular effects of TGF-β1 begin with its binding to the high-affinity cell-surface TGF-β type I [TβRI, or activin-like receptors (ALK receptors)], type II (TβRII) or type III (TβRIII) receptors. TGF-β1 binds to TβRIII, a complex that is then presented to TβRII. Alternatively, TGF-β1 can binds directly to TβRII. Once TGF-β1 binds to TβRII, this receptor transiently phosphorylates TβRI (ie. induce its activation) (1,3,25).

In the TGF-β1 signalling transduction pathway, Smads play an essential role in mammalian cells. These proteins are derived from the homologous of genes *sma* and *Mad* in *C. Elegans* and *D. Melanogaster,* respectively. Smads are grouped in three subfamilies: (*i*) Receptors-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, Smad8) which

are phosphorylated by TβRI, (*ii*) Common mediator Smads (Co-Smad: Smad4) which binds with active R-Smads, and (*iii*) Inhibitory Smads (I-Smads: Smad6, Smad7) (25). TβRI phosphorylates Smad2 or Smad3, which bind to Smad4 and enter to the nucleus, where the complex Smad2/3/4 interacts with other transcription factors, coactivators and corepressors to modulate transcription of several genes. On the other hand, TGF-β1 can activate Smad6 and Smad7. Smad6 inhibits BMP signalling pathways, whereas Smad7 inhibits TGF-β1 as well as BMP signalling pathways. Smad6 and Smad7 lack of the region susceptible to phosphorylation present in R-Smads, but they have the capacity to bind to TβRI preventing R-Smads phosphorylation (1,25).

TGF-β1 and the L-Arginine/Nitric Oxide Signalling Pathway

The L-Arginine/NO Pathway

The NO is a co-product derived from the conversion of L-arginine into L-citrulline catalysed by NO synthases (NOS). The nitric oxide (NO) is a gas that diffuses into vascular smooth muscle cells (VSMC), where activates the soluble guanylyl cyclase leading to vasodilatation (8,16,21). There are at least three NOS isoenzymes coded by independent genes: neuronal (nNOS or NOS I), inducible (iNOS or NOS II) and endothelial (eNOS or NOS III). The capacity of endothelial cells to synthesise NO is determined by the level of eNOS expression, modulation of its catalytic activity by Ca^{+2}/calmodulin, and the bio-availability of essential cofactors (ie. BH_4, NADPH, FAD, FMN), oxygen and its substrate L-arginine (8). The activity of eNOS seems to be associated with an efficient uptake of L-arginine in several cell types (2,4,6,13,14,21,28, 30) (**Fig. 1**). The membrane transport systems for cationic amino acids described in endothelium include at least four different groups: system y^+, recognized as a family of cationic amino acid transporters, CAT (ie. system y^+/CAT), with at least five members; system y^+L, with high affinity for the Na^+-dependent exchange of cationic amino acids by neutral amino acids; system $b^{0,+}$, Na^+-independent, and system $B^{0,+}$, Na^+-dependent (5,13,21). In primary cultures of human umbilical vein endothelial cells (HUVEC), L-arginine transport is mediated by human CAT-1 (hCAT-1) and hCAT-2B isoforms (13,21,27,28). It has been demonstrated that transport activity via hCAT1 or hCAT-2B, or both (13,21,27), and system y^+L (2) is crucial for the synthesis of NO in HUVEC and in endothelial cells from human placental microvessels (7). There are studies suggesting that the co-localization of eNOS and CAT-1 in caveolae may facilitate the “coupling” (ie. NO synthesis activity) of eNOS (14), agreeing with the proposed ‘L-arginine compartmentalization’ hypothesis (4,21).

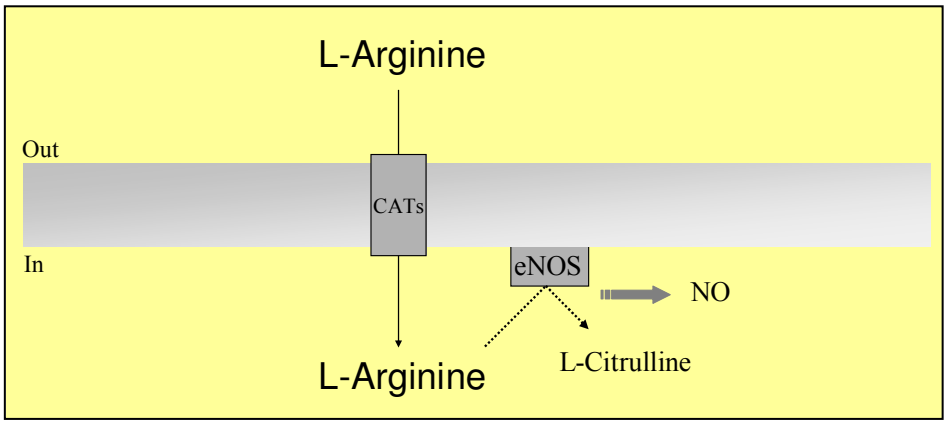

Figure 1. L-Arginine/Nitric Oxide pathway in endothelial cells. *L-Arginine is taken up by Cationic Amino acid Transporters (CATs) and metabolize via the endothelial nitric oxide synthase (eNOS) to produce L-citrulline and NO.*

TGF-β1 and L-Arginine transport

There are only few studies reporting the effect of TGF-β1 on L-arginine transport. Interestingly, studies by Oyadomari *et al.* (18) show that experimental diabetes is associated with higher expression of TGF-β1, but without alterations in the mRNA level for cationic amino acid transport system y^+ in rat aorta. Unfortunately, this study was not focused in the determination of kinetic properties of cationic amino acid transport in this preparation. Durante *et al.* (6) showed that exposure of rat aortic VSMC to TGF-β1 increases L-arginine transport in a concentration-dependent manner. The increase of L-arginine transport was associated with greater maximal velocity (V_{max}), an effect that was abolished by cycloheximide (inhibitor of protein synthesis), suggesting that TGF-β1 effect requires *de novo* protein synthesis, which could correspond to the proper membrane transporters or proteins acting as activators of a pre-existent pool of cationic amino acid transporters. In addition, TGF-β1 effect on L-arginine transport could be explained by increased CAT-1 expression, since mRNA level for this isoform was increased ~6-fold, without significant changes in CAT-2B mRNA level. However, these authors also show that TGF-β1 effect on L-arginine transport was associated with a higher absolute value of the apparent K_m. This result suggest that increased L-arginine transport could also be due to altered intrinsic properties of these membrane transporters, such as structural modifications or altered kinetics for substrate-carrier complex formation and/or separation, among other factors (5,9). Preliminary studies in primary cultures of HUVEC show that TGF-β1 increases the V_{max} and the transport capacity (ie. V_{max}/K_m) for L-arginine transport (28). This effect of TGF-β1 was associated with a higher number of copies for hCAT-1 mRNA and increased phosphorylation of $p42/44^{mapk}$ and Smad2. Confirming the involvement of TβRII on the TGF-β1 effect on L-arginine transport are the results showing that overexpression of a truncated form of TβRII in HUVEC block the effect of TGF-β1. However, it is interesting to emphasize that TGF-β1 effect on L-arginine transport kinetics characterization in endothelial cells has not been fully studied (for reviews see 5,13,21).

TGF-β1 and NO synthesis

eNOS expression can be regulated by transcriptional, post-transcriptional and post-translational mechanisms. In the 5'-flanking region of the eNOS gene, consensus sites for Sp-1, AP-1, PEA3 and GATA have been detected (20). Several studies have described that TGF-β1 increases eNOS expression (10,18,22,28). It has been found that incubation of bovine aortic endothelial cells (BAEC) with TGF-β1 increased eNOS mRNA, protein and activity. TGF-β1 regulates eNOS expression in part by increasing the binding of nuclear factor 1 (NF-1) to eNOS promoter (10). TGF-β1 also increased the level of eNOS mRNA in human aortic endothelial cells (HAEC) and eNOS protein abundance in HUVEC (22). Induction of eNOS expression by TGF-β1 is mediated by Smad2 which binds to eNOS promoter (22).

Recently, it has describes the presence of TβRI y TβRII receptors in caveolae in HUVEC colocalizing with eNOS and regulating its function (23). Short periods of incubation with TGF-β1 (5-15 minutes) reduced immunoprecipitation of eNOS with TβRI

and TβRII, L-citrulline production from L-arginine, and eNOS phosphorylation in Ser^{1177}. However, Schwartz and colleagues did not find changes in eNOS subcellular distribution by electronic microscopy in response to TGF-β1. Unfortunately, these authors did not check whether longer times of exposure to TGF-β1 altered expression and activity of eNOS.

In a rat model of septic shock induced by lypopolysacharide (LPS), TGF-β1 decreased iNOS mRNA and protein level in heart, kidney, lung, liver and spleen, accompanied with lower hypotension and mortality associated with this disease (19). TGF-β1 also decreased iNOS expression in rat aortic VSMC. However, TGF-β1 does not change eNOS mRNA level in heart, kidney and lung. TGF-β1 is beneficial in myocardic ischemia and reperfusion models, a condition associated with endothelial dysfunction (12). TGF-β1 has been shown to decrease superoxide anion ($O_2^{\bullet}$) levels in coronary circulation, maintaining endothelium-dependent vasodilatation, and decreasing the damage produced by TNF-α in rats (12). In a splanchnic artery occlusion followed by reperfusion in cat, TGF-β1 increases endothelium-dependent vasodilatation (11). These *in vivo* studies confirm that TGF-β1 is directly involved in the modulation of vascular tone maintaining an appropriate NO synthesis by the endothelium.

TGF-β1 also inhibits the release of NO derived from iNOS in a concentration-dependent manner in a rat cerebral microvascular endothelial cell line (17). In addition, Ungureanu-Longrois *et al.* (26) described that rat cardiac microvascular endothelial cells have a low eNOS activity. Interestingly, these cells respond to a combination of inflammatory mediators (IL-1β and IFN-γ) with an increase of iNOS activity, an effect that is significantly decreased by TGF-β1 (26). Changes in iNOS expression induced by TGF-β1 are explained by a lower transcription, mRNA stability, mRNA translation and protein stability (29).

On the other hand, several studies show that NO could alter TGF-β1 levels in different cell types. Recently, it was described in HUVEC that NO inhibits TGF-β1/Smads signalling, in a cGMP-dependent manner. NO decreased phosphorylation of Smad2 in the nucleus and increased ubiquitinization, decreasing Smad2 half-life (1,3,22). In mesangial cells, exogenous NO derived from *S*-nitroso-L-acetyl-L,D-penicillamine (SNAP) or endogenous NO induced by IL-1β, decreased TGF-β1 activity and the elevation of TGF-β1 mRNA level and collagen synthesis in cells exposed to high concentrations of D-glucose or phorbol esters (24). Aorta from eNOS *knockout* ($eNOS^{-/-}$) mice exhibits increased TGF-β1 expression (22). In addition, in $eNOS^{-/-}$ rat derived aortic endothelial cells there is an increased Smad2 phosphorylation and transcriptional activity. Thus, reduced NO seems to increase the expression and cell signalling pathways activated by TGF-β1.

Concluding remarks

Figure 2 summarises the potential effects of TGF-β1 in endothelial cells. TGF-β1 signalling in endothelial cells implies activation of TβRII leading to increased Smad2 and $p42/44^{mapk}$ activation. This effect of TGF-β1 results in increased expression of hCAT-1 membrane transporters since its activity was increased by this growth factor. TGF-β1 also seems to increase NO synthesis as a consequence of higher expression of eNOS in endothelial cells; however, since NO is able to downregulate expression of TGF-β1 these results must be taken with caution. We suggest that NO increased by TGF-β1, at least in

HUVEC, may be one of the steps in a cycle where increased level of NO regulates TGF-β1 expression, as described for NO modulation of eNOS expression in several cell types.

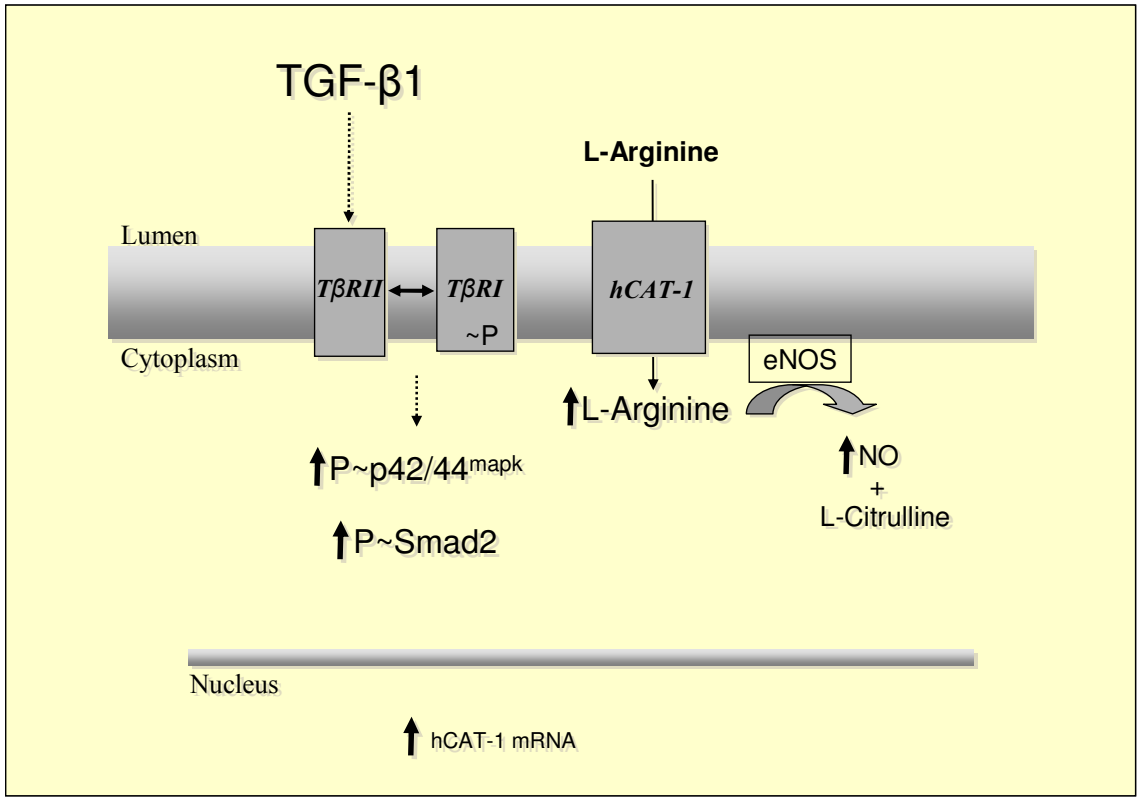

Figure 2. Stimulation of endothelial L-arginine/nitric oxide pathway by TGF-β1. *TGF-β1 binds to TβRII receptor, inducing phosphorylation (~P) of TβRI receptor leading to increased phosphorylation (ie. activation) of the signalling molecules p42/44^{mapk} (P~p42/44^{mapk}) and Smad2 (P~Smad2). TGF-β1 stimulates L-arginine transport and increases the number of copies for hCAT-1 mRNA. TGF-β1 also stimulates endothelial nitric oxide synthase (eNOS) expression and activity.*

Since diabetic patients exhibit increased plasma levels of TGF-β1 and it has been shown that these patients also have increased NO levels in the early stages of the disease, it is interesting to consider the possibility that abnormally elevated plasma levels of D-glucose could induce release of TGF-β1 (15,18,28) leading to stimulation of the L-arginine/NO signalling pathway in endothelial cells.

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XXII CONGRESO LATINOAMERICANO Y 1ER IBERO-AMERICANO DE CIENCIAS FISIOLÓGICAS

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Buenos Aires, 4 al 7 de noviembre de 2006

Este año tendrá lugar en Buenos Aires el XXII Congreso de la Asociación Latinoamericana de Ciencias Fisiológicas (ALACF). Esta reunión congregará a científicos originarios de América Latina trabajando en sus países de origen, en Estados Unidos, en Europa y alrededor del mundo. Fisiólogos no latinoamericanos de primer nivel son también regularmente invitados. Esta vez la Sociedad Española de Ciencias Fisiológicas se asocia al evento, dándole especial interés y relevancia.

El objetivo central del Congreso es dar, a los fisiólogos trabajando y viviendo en Latinoamérica, la posibilidad de entrar en contacto con referentes en su campo de trabajo. Esto será especialmente cierto esta vez para aquellos radicados en el Cono Sur del continente (Bolivia, Brasil, Chile, Paraguay, Uruguay y Argentina).

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